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PREPARATION OF CALCIUM-SENSITIVE α-CASEIN

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SUMMARY

A relatively simple modification of the method for fractionating whole casein in urea solutions is described which gives a calcium-sensitive α-casein. The yield is about 40% of total α-casein. This casein and α-paracasein precipitate to the same extent with $CaCl_2$; 10 m*M* $CaCl_2$ per liter at pH 7 is sufficient to precipitate a 1% solution of each casein. The two caseins can, however, be distinguished by their initial rates of aggregation with lower concentrations of $CaCl_2$ (1.5 to 3.5 m*M* per liter). With low concentrations of $CaCl_2$, whole α-casein aggregates to a greater extent than does calcium-sensitive α-casein. The calcium-sensitive α-casein appears to be homogeneous on electrophoresis at pH 8.5 and 2.3. Very little (less than 1%) of this casein is made soluble by the action of rennin.

The preparation of calcium-sensitive and calcium-insensitive (kappa) casein has been described by Waugh and vonHippel (9). The procedure required an ultracentrifuge. The present investigation was begun when α-caseins, prepared by different individuals by differential solubility in 4.6 *M* urea (4), were found to differ greatly in their sensitivity to calcium ion. In one instance, the product was precipitated almost quantitatively at pH 7.0 with 10 m*M* $CaCl_2$ per liter, similarly to a product prepared by the method of Waugh and vonHippel (9). In other instances, less than 40% of the α-casein was precipitated when the concentration of $CaCl_2$ was as much as 200 m*M* per liter. This paper describes the conditions for preparing either product. When the calcium-sensitive α-casein is prepared, a fraction rich in the calcium-insensitive α-casein is obtained. The method for characterizing the caseins by their quantitative precipitation with $CaCl_2$ is described.

EXPERIMENTAL PROCEDURE AND RESULTS

Preparation of whole casein. The casein was precipitated from skimmilk by acidification to pH 4.5 with *N* HCl. The precipitate was washed four times with water and twice dissolved and reprecipitated with acid (3). The precipitated casein was extracted and dried by washing successively with ethanol, acetone, and ether.

Preparation of α-casein. The α-casein, which will be called whole α-casein, was prepared from casein by differential precipitation from urea solutions (4). As a rule, unpurified acid-precipitated casein, stored in frozen condition, served as the starting material. The precipitate was suspended in water, washed several times with water, and dissolved in urea solution. The procedure described by Hipp *et al.* (4) was followed. The final removal of the urea from the casein precipitate by washing with water was facilitated by first breaking up the sticky precipitate suspended in water in a Waring blendor. The final product

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was dried with organic solvents in the same manner as the whole casein. As a rule, the products obtained by this method, although showing some variation, were relatively insensitive to $CaCl_2$ in the precipitation test described later.

Preparation of calcium-sensitive α-casein. Use of the above method for preparing α-casein led in one instance to a product that was very sensitive to $CaCl_2$ (90% precipitated with 10 m*M* $CaCl_2$ per liter). The major variation in the procedure that led to the calcium-sensitive product was the use of decantation instead of centrifugation to separate the α-casein precipitated by diluting the 6.6 *M* urea solution with water to 4.6 *M* urea concentration. This procedure, however, did not always give a calcium-sensitive α-casein (2). Calcium-sensitive products also were occasionally obtained when the precipitate obtained in 3.3 *M* urea was kept at 7° for 18 hr. Similarly, the addition of a small amount of NaOH (6 ml. of 0.1 *N* for 150 g. of wet casein) at this stage in the fractionation sometimes led to a calcium-sensitive product. Calcium-sensitive α-casein was regularly obtained, however, with the following simple modification of the fractionation in urea solutions (4). One hundred and fifty grams (approximately 32 g. dry weight) of wet casein were fractionated at one time. Following the first precipitation (4.6 *M* urea), the concentration of NaCl in the 6.6 *M* urea was doubled from the 1.59 g. used by Hipp *et al.* (4) to 3.18 g. per 150 ml. The α-casein was precipitated (A) with 150 ml. of water in the usual way. The part remaining in solution was recovered to give a calcium-insensitive fraction for future study. The α-casein precipitate (A) was dissolved again in 6.6 *M* urea containing 3.18 g. NaCl per 150 ml. and precipitated again by the addition of water. This product was washed free of urea as described for whole casein, and extracted with the organic solvents. Yield: 7.5 to 8.0 g.

Preparation of α-paracasein. The α-paracasein was prepared by the action of pepsin on α-casein at pH 6.5 in the presence of 15 m*M* $CaCl_2$ per liter. Solution of the calcium-precipitated paracasein was facilitated by the addition of Versene equivalent to the calcium present, and the Ca-versenate was eliminated by reprecipitation at pH 4.7 and dialysis. Other details of the procedure have been described (12).

Precipitation of caseins with calcium chloride. The degree to which casein precipitates with calcium chloride is an important property of casein and it is desirable to characterize each preparation by its quantitative precipitation with a range of $CaCl_2$ concentrations. A test of this type has been described by Nitschmann *et al.* (1, 6). These authors added $CaCl_2$ to a 3% solution of casein. In the present studies, a 1% solution of casein was used and the test has been modified in other minor ways. Each test was performed with several concentrations of $CaCl_2$. These were chosen within the range of 2 to 200 m*M* per liter, depending on the expected calcium-sensitivity of the casein under test. The final solution contained 1% casein, adjusted to pH 7.0 with NaOH and a known concentration of $CaCl_2$ in a test volume of 10 ml. The solution was placed in a bath for 1 hr. at 30° C., then centrifuged for 10 min. at approximately 3,000 × G (International centrifuge, Head No. 233, 15-ml. centrifuge tube, rheostat reading 40). A portion of the supernatant solution was removed and the casein de-

termined by measuring the light absorption at 280 mμ, after clarifying by adding NaOH, or by binding the calcium ion with versene (11). The results obtained with several casein preparations are shown in Figure 1.

Electrophoresis of caseins. Calcium-sensitive caseins had the same mobility as the original α-casein at pH 8.6 on electrophoresis by the Tiselius technique. Electrophoresis at pH 2.3 (5) provides more information about whole α-casein and its fractions. The whole α-casein, prepared by the original urea method of Hipp *et al.* (4), at pH 2.3, shows a major peak with a shoulder on the slow side and also a small amount (about 5%) of a component with a considerably smaller mobility. Electrophoresis of the calcium-sensitive α-casein at pH 2.3 shows only a single symmetrical peak.

Rate of aggregation of the caseins with calcium chloride. Although the calcium-sensitive α-casein, isolated by the present procedure, and paracasein prepared from whole α-casein, both precipitate almost quantitatively with 10 m*M* $CaCl_2$ per liter (see Figure 1), the two are not identical. Experimentally, the two preparations (calcium-sensitive α-casein and α-paracasein) can be dis-

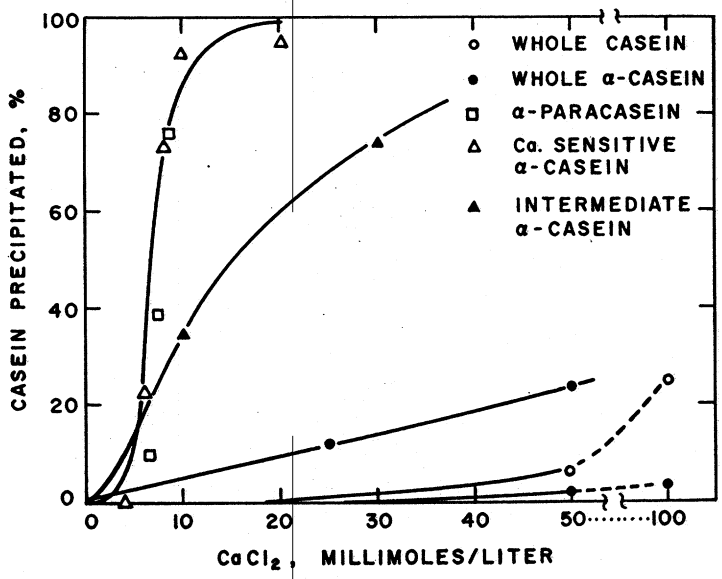

FIG. 1. Precipitation of various casein preparations with calcium chloride at pH 7.0. Results shown for two whole α-casein preparations show the variation observed with the regular procedure.

tinguished by their sensitivity to low concentrations of calcium ions (1.5 to 3.5 m*M* per liter), and the initial course of their aggregation in the presence of calcium ions. Previous spectrophotometric studies (12) have shown that whole α-casein in the presence of $CaCl_2$ will aggregate in a linear manner for more than 30 min. Paracasein, on the other hand, aggregates very rapidly in the first few minutes, then much more slowly, giving a rectilinear type of curve (12).

α-Paracasein, prepared either from whole α-casein or calcium-sensitive α-casein, gives a similar type of aggregation curve, although less exaggerated, as shown in Figure 2. Also shown in the same figure, calcium-sensitive α-casein gives a linear aggregation curve, similar to whole α-casein.

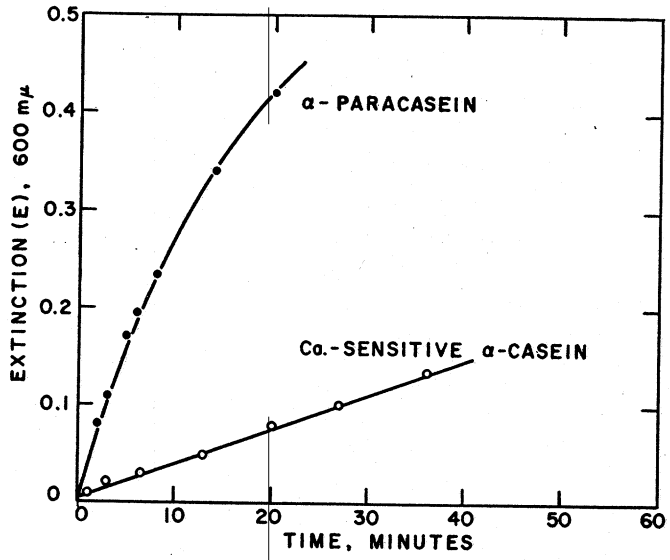

FIG. 2. Rate of aggregation of α-paracasein and calcium-sensitive α-casein in the presence of calcium ions, measured by the light extinction (E) at 600 mμ. The reaction was performed in cacodylate buffer, pH 6.3 at 30° (12). ● : α-paracasein with 1.5 m*M* $CaCl_2$ per liter; ○: Calcium-sensitive α-casein with 3.3 m*M* $CaCl_2$ per liter.

α-Paracasein in the above type of rate experiment is much more sensitive to calcium ion concentration than is the calcium-sensitive α-casein. The respective concentrations used for the experiment illustrated in Figure 2 are 1.5 and 3.3 m*M* $CaCl_2$ per liter. If these preparations and whole α-casein are all compared at pH 6.3 with the same $CaCl_2$ concentration of 3.3 m*M* per liter, an extinction of 0.5 is attained by α-paracasein in 0.5 min., whole α-casein in 10 min., and calcium-sensitive α-casein in more than 2 hr. Because of the difference between whole α-casein and calcium-sensitive α-casein, just the reverse of that expected from precipitation experiments, aggregation experiments were done with both caseins with 3 to 6 m*M* $CaCl_2$ per liter at pH 7.2. Since the aggregate obtained with whole α-casein is stable, the ultracentrifuge was used (45 min. at 105,000 × G) to sediment the aggregates. Neither casein aggregated at this pH (7.2) and temperature (25° C.) with 3 m*M* $CaCl_2$ per liter (at a higher temperature only the whole α-casein became opalescent); with 4 m*M* only 10% of sediment was obtained with the calcium-sensitive α-casein, whereas 22% of sediment was obtained with the whole α-casein. The sedimentation curves crossed between 5.5 and 6.0 m*M*, and at 6 m*M* the respective amounts of sediment were 71 and 63% of the total casein.

The heat-induced increase in the calcium ion aggregation of calcium-sensitive α-casein at lower temperature shows the same type of reversal of aggregation as whole α-casein (10); that is, the higher the initial temperature, the greater the decrease in aggregation on going to a lower temperature. Paracaseins in a similar test reverse very little.

Other properties of calcium-sensitive α-casein. The preparations of calcium-sensitive α-casein obtained by the present procedure regularly contained more phosphorus than whole α-casein. The analysis of a calcium-sensitive preparation was as follows (all on moisture-free basis): P, 1.08%; N, 15.16%; ash, 4.31%.

The various casein preparations were also characterized by the amount that remained soluble when the solution was adjusted to pH 4.7 after rennin had acted on the casein at pH 6.4. The procedure employed and the results have been described (2). The net increase in the soluble portion of whole α-casein was 2.8 to 3.5%, whereas for the calcium-sensitive α-casein, it was only 0.64 to 0.80%.

DISCUSSION

The method just described gives a calcium-sensitive α-casein in relatively good yield, by a simple modification of the frequently used fractionation of whole casein in urea solutions. Further, the effectiveness of the additional NaCl in separating the calcium-sensitive and calcium-insensitive fractions of α-casein suggests that the interaction between these components, at least in part, is due to interaction of electrical charge on the proteins. Increasing the concentration of the salt ion in the environment will reduce such an interaction.

The calcium chloride precipitation test described herein provides a useful characterization for each preparation of whole casein and α-casein. In view of the contribution of the stabilizing fraction to the stability in the presence of calcium ions, it is not surprising that there is some variation in precipitation with $CaCl_2$ from one preparation of casein to another. The $CaCl_2$ test can provide an important characterization for each preparation until the time when a method is available for determining the calcium-stabilizing α-casein content of each sample of milk or preparation of casein. This is even more true of the whole α-casein preparations, since the present study shows that fractionation in urea solutions is sensitive to minor variations in the procedure. If the procedure is standardized, the product will be of constant properties and presumably constant composition, but it is recommended that the $CaCl_2$ test be performed for confirmation.

The phosphorus content for calcium-sensitive casein obtained in the present study is about the same as the 1.1% reported by Waugh (8) for α_s-casein, a calcium-sensitive casein. In the present study, casein made by the procedure of Waugh and vonHippel (9) precipitated with $CaCl_2$ in the same way as the calcium-sensitive casein illustrated in Figure 1. McMeekin *et al.* (5) have shown that a calcium-sensitive casein with about the same phosphorus content and apparently homogeneous on electrophoresis at pH 2.35 could be fractionated in ammonium sulfate solutions to give a product containing 0.85% phosphorus (α_1-casein) and a fraction correspondingly richer in phosphorus.

The amount of casein becoming soluble when it is acted on by rennin can serve to guide the separation of calcium-sensitive and calcium-insensitive products. The former gives very little material soluble with rennin, whereas the latter containing the stabilizing fraction gives a considerable amount. Wake (7) reported that none of a purified preparation of calcium-sensitive casein became soluble when acted on by rennin. The method for preparing this casein was not described. The small amount becoming soluble in the present study may be due to contamination with the stabilizing fraction. This seems unlikely, since the casein was reprecipitated in urea under conditions for separating the stabilizing fraction and the product still gave about the same amount soluble with rennin. An alternative explanation may be that in the rennin test some nonspecific proteolysis may have occurred, not related to the role of the stabilizing fraction in clotting.

The greater amount of aggregate obtained with whole α-casein at low calcium chloride concentrations (about 4 m*M* per liter at pH 7.2) than with calcium-sensitive α-casein is an interesting phenomenon. When aggregates are obtained with the latter, they coagulate and settle, whereas the former are stable colloids. The explanation for the differences noted is not apparent, but the calcium-sensitive α-casein may bind more calcium ion per unit weight than the whole α-casein and only when saturated with calcium ion is the unstable aggregate formed.

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